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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	09/600,130	Confirmation No.. 8898
Applicant	:	Keith Campbell	
Filed	:	May 14, 2001	
TC/A.U.	:	1600/1632	
Examiner	:	Joseph T. Woitach	
Title	:	Double Nuclear Transfer Method and Results Thereof	
Docket No.	:	10758.105003 REV1001	
Customer No.	:	20786	

Mail Stop PETITION
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

RENEWED PETITION UNDER 37 CFR 1.181

Sir:

In response to the Decision on Petition ("Decision") mailed November 15, 2006 in the referenced application, for which a request for reconsideration is due January 15, 2007, please consider the following remarks.

The Applicant named in the above-identified patent application previously petitioned under 37 CFR § 1.181(a) to withdraw the holding of abandonment made by the Examiner in the instant application. Specifically, the Applicant requested withdrawal of the holding of abandonment made in the Notice of Abandonment ("Notice") mailed on March 23, 2006. The Applicant attaches hereto a copy of the July 28, 2006 Petition to Withdraw Holding of Abandonment Under 37 CFR 1.181(a).

In the Decision, the Office of Petitions has dismissed the aforementioned petition to withdraw the holding of abandonment in light of 37 CFR 1.8(b)3. It is understood that before the Patent Office can withdraw the holding of abandonment, a request for reconsideration and a statement in compliance with 37 CFR 1.8(b)3 must be submitted. The required statement follows:

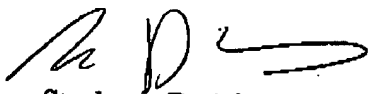
Appl. No. 09/600,130
Keith Campbell
Renewed Petition Under 37 CFR 1.181

STATEMENT: I hereby attest on a personal knowledge basis to the September 13, 2005 mailing of a response to the April 13, 2005 Office Letter in the instant application. I am the person who signed the Certificate of Mailing in the original response.

Applicant respectfully requests reconsideration of the Decision.

No fees are believed to be due in connection with this Renewed Petition under 37 CFR 1.181. However, the Commissioner is authorized to charge any underpayment of fees to Deposit Account No. 11-0980.

Respectfully submitted,



Stephanie D. Adams, Ph.D.
Patent Agent
Registration No. 47,378

Date: November 28, 2006

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Mail Stop PETITION
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

PETITION TO WITHDRAW HOLDING OF ABANDONMENT UNDER 37 CFR 1.181(A)

Sir:

The Applicant named in the above-identified patent application hereby petitions under 37 CFR § 1.181(a) to withdraw the holding of abandonment made by the Examiner in the instant application. Specifically, the Applicant requests withdrawal of the holding of abandonment made in the Notice of Abandonment ("Notice") mailed on March 23, 2006.

In the Notice, the Examiner states that this application is abandoned in view of the "Applicant's failure to timely file a proper reply to the Office Letter mailed April 13, 2005." Further, the Examiner states that "no reply has been received." However, the Applicant disagrees with this holding on the basis that there is no abandonment in fact.

There is no abandonment in fact because a response to the April 13, 2005 Office Letter was mailed to the Patent Office on September 13, 2005, with a certificate of mailing declaration as part thereof in accordance with MPEP § 512.

The Applicant attaches hereto a postcard receipt stamped by the Office of Initial Patent Examination ("OIPE") evidencing that the response mailed on September 13, 2005 was in fact received by the Patent Office on September 15, 2005, as indicated by the OIPE date stamp on the lower right-hand corner of the postcard receipt.

In addition to the postcard receipt, the Applicant is also furnishing an entire courtesy copy of the contents of the response that was filed and that is detailed on the aforemen-

tioned postcard receipt. Specifically, the Applicant is attaching to this Petition one copy of each of the following documents:

(1) Response and Amendment to Office Action (9 pp.);

(2) Request for Extension of Time (1pp.);

(3) Check No. 484685 for $225 (1 pp.);

(4) Postcard;

(5) Article entitled "Evidence of a Pluripotent Human Embryonic Stem Cell Line Derived from a Cloned Blastocyst" by Hwang et al. (6 pp.);

(6) Article entitled "Comment on Molecular Correlates of Primate Nuclear Transfer Failures" by Lanza et al. (1 pp.);

(7) Article entitled "Somatic Cell Nuclear Transfer (Cloning) Efficiency" by Paterson (5 pp.);

(8) Article entitled "Dogs Cloned from Adult Somatic Cells" by Lee et al. (1 pp.);

(9) Article entitled "Potential for Cloning Dogs" by Westhusin et al. (7 pp.); and

(10) Certificate of Mailing (1 pp.) evidencing that the contents described therein were timely submitted on September 13, 2005.

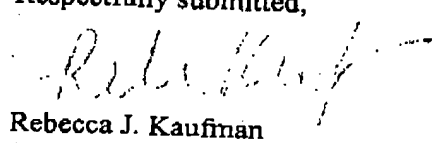
In view of the above, the Applicant requests reconsideration and withdrawal of the holding of abandonment. Further, the Applicant requests consideration and entry of the attached timely filed Office Action response.

No fees are believed to be due in connection with this Petition to Withdraw Holding of Abandonment. However, should the Commissioner determine otherwise, he is authorized to charge any underpayment of fees to Deposit Account No. 11-0980.

If the Examiner believes that there are any issues that can be resolved by a telephone conference, or that there are any formalities that can be corrected by an Examiner's amendment, please contact the undersigned at (404) 572-4600.

Date: July 28, 2006

Respectfully submitted,



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Rebecca J. Kaufman
Reg. No. 44,819

The U.S. Patent and Trademark Office official mailroom stamp affixed hereto acknowledges receipt of the items listed below.

Date Mailed September 13, 2005

Applicant : Keith Campbell

Appl. No. : 09/600,130

Filed : May 14, 2001

Title: Double Nuclear Transfer Method and Results Thereof

Papers Submitted: Response and Amendment to Non-Final Office Action (9pp.) Request for Extension of Time, Fee Transmittal, Check for $225.00, Certificate of Mailing, and Postcard

Attorney/Agent: Stephanie D. Adams

Docket No.: 10758.105003 REV1001

3829258_1.DOC

PATENTS

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Appl. No.	:	09/600,130
Applicant	:	Keith Campbell
Filed	:	May 14, 2001
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Examiner	:	Joseph T. Woitach
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Docket No.	:	10758.105003
Customer No.	:	20786

Confirmation No. 8898

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

September 13, 2005

RESPONSE AND AMENDMENT TO OFFICE ACTION

Sir:

In response to the Office Action of April 13, 2005, for which a response at the two month deadline is due on September 13, 2005, please amend the above-identified applications as follows:

Amendments to the Claims begin on page 2 of this paper.
Remarks/Arguments begin on page 6 of this paper.

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Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claims 1-41 (canceled)

Claim 42 (previously presented): A method of reconstituting an animal embryo, comprising

(i) transferring a donor nucleus from a diploid somatic cell into a first recipient oocyte;

(ii) removing the nucleus from the first recipient oocyte;

(iii) either activating a second recipient oocyte or enucleating a fertilized zygote; and

(iv) transferring the nucleus from the first recipient oocyte into the preactivated second recipient oocyte or the enucleated fertilized zygote.

Claim 43 (previously presented): The method as claimed in claim 42, wherein the first oocyte is a mature metaphase II oocyte or an activated metaphase II oocyte.

Claim 44 (previously presented): The method as claimed in claim 42, wherein the second oocyte is an enucleated metaphase II oocyte.

Claim 45 (previously presented): The method as claimed in claim 42, in which a reconstructed embryo obtained thereby is cultured *in vitro* or *in vivo* to a stage suitable for transfer to a final surrogate recipient for development to term.

Claim 46 (previously presented): The method as claimed in claim 42, in which a reconstructed embryo obtained thereby is transferred to a final surrogate recipient to support embryo development and development to term.

Claim 47 (previously presented): The method as claimed in claim 42, in which the donor nucleus is genetically modified.

Claim 48 (canceled)

Claim 49 (previously presented): The method as claimed in claim 42, wherein the donor nucleus is from a G1 cell.

Claim 50 (previously presented): The method as claimed in claim 42, wherein the diploid cell is arrested at the G1/S-phase border.

Claims 51-55 (canceled)

Claim 56 (previously presented): The method as claimed in claim 42, wherein the donor nucleus is donated by a diploid cell arrested by any point in the cell cycle.

Claim 57 (previously presented): The method as claimed in claim 42, wherein the first recipient oocyte is enucleated.

Claim 58 (previously presented): The method as claimed in claim 42, wherein the donor nucleus is transferred into the first recipient oocyte by cell fusion, or by cell or nuclear injection.

Claim 59 (previously presented): The method as claimed in claim 42, in which the animal embryo is an ungulate species embryo.

Claim 60 (previously presented): The method as claimed in claim 59, wherein the animal embryo is a cow or bull, pig, sheep, goat, camel, or water buffalo embryo.

Claim 61 (previously presented): The method as claimed in claim 42, wherein the animal embryo is a mouse, rat, or other rodent embryo.

Claim 62 (previously presented): The method as claimed in claim 42, wherein the animal embryo is a lagomorph embryo.

Claim 63 (previously presented): The method as claimed in claim 62, wherein the animal embryo is a rabbit embryo.

Claims 64-65 (canceled)

Claim 66 (previously presented): The method as claimed in claim 42, wherein the nucleus is transferred from the first recipient oocyte to a fertilized zygote.

Claim 67 (previously presented): The method as claimed in claim 42, wherein the second recipient oocyte is activated by chemical or physical means.

Claim 68 (previously presented): The method as claimed in claim 42, wherein the second recipient oocyte is enucleated.

Claim 69 (previously presented): A method of preparing an animal, the method comprising:

(a) reconstituting an animal embryo as claimed in claim 42, thereby obtaining a reconstituted embryo,

(b) causing a fetus to develop from the embryo, thereby obtaining an animal a fetus; and

(c) causing an animal to develop to term from the animal a fetus, thereby obtaining an animal.

Claim 70 (previously presented): The method as claimed in claim 69, further comprising:

(d) breeding the animal.

Claim 71 (previously presented): A method as claimed in claim 69, wherein the animal embryo is further manipulated prior to full development of the embryo.

Claim 72 (previously presented): A method as claimed in claim 69, wherein the animal fetus is further manipulated prior to full development of the fetus.

Claim 73 (previously presented): The method as claimed in claim 69, wherein a new cell line or cell population is derived from the reconstituted embryo.

Claim 74 (previously presented): The method as claimed in claim 69, wherein a new cell line or cell population is derived from the animal fetus.

Claim 75 (previously presented): The method as claimed in claim 69, wherein a new cell line or cell population is derived from the animal.

Claim 76 (previously presented): The method as claimed in claim 69, wherein more than one animal is derived from the reconstituted embryo.

Claims 77-82 (canceled)

Claim 83 (previously presented): The method as claimed in claim 67, wherein the chemical or physical activation is by a treatment that induces calcium entry into the oocyte or release of internal calcium stores.

Claim 84 (previously presented): The method as claimed in claim 67, wherein the chemical activation is by treatment with ethanol, ionomycin, inositol tris-phosphate or calcium ionophore A23187.

Claim 85 (previously presented): The method as claimed in claim 67, wherein the chemical activation is by treatment with extracts of sperm.

Claim 86 (previously presented): The method as claimed in claim 67, wherein the physical activation is by application of a DC electrical stimulus.

Claim 87 (previously presented): The method as claimed in claims 83-86, wherein the chemical or physical activation further comprises treatment with inhibitors of protein synthesis or inhibitors of serine threonine protein kinases.

Claim 88 (previously presented): The method as claimed in claim 42, wherein the animal embryo is a pig embryo.

Claims 89 (canceled)

Claim 90 (previously presented): The method as claimed in claim 59, wherein the animal embryo is a cow embryo.

Claim 91 (previously presented): The method as claimed in claim 59, wherein the animal embryo is a pig embryo.

Claim 92 (previously presented): The method as claimed in claim 59, wherein the animal embryo is a sheep embryo.

Claims 93-113 (canceled)

REMARKS/ARGUMENTS

After entry of this amendment claims 42-47, 49, 50, 56-63, 66-76, 83-88 and 90-92 remain pending in this application. Applicants have canceled claims 89 and 93-113 without prejudice. Applicants reserve the right to pursue such claims in a continuation or divisional application.

Claims 89 and 93-113

Claims 89 and 93-113 were objected to under 37 CFR 1.75 as being a substantial duplicate of claims 42-47, 49, 50, 56-63, 66-68, and 90-92. On the contrary, Applicants can claim their invention with any wording they choose, as it is a matter of opinion whether a claim is a substantial duplicate. Claims 89 and 93-113 were also rejected under 35 USC § 112 for lacking written description for methods to generally make a cell. Applicants note that the specification provides an ample description of methods to make cells, including, embryonic cells, such as stem and germ cells, which can be differentiated into various cell populations, for example, but not limited to, on page 1 (lines 15-20) and page 3 (lines 10-23) of the specification. However, to facilitate prosecution and allowable subject matter, Applicants have canceled claims 89 and 93-113 and intend to file a continuation application to pursue these claims.

Claims 42-47, 49, 50, 56-63, 66-76, and 83-113:
Rejection Under 35 U.S.C. § 112, 1st Paragraph

The Examiner has rejected claims 42-47, 49, 50, 56-63, 66-76, 83-113 under 35 U.S.C. § 112, first paragraph as lacking enablement based on the Examiner's assertion that undue experimentation is required to practice the full scope of the claims. Claims 89 and 93-113 were also rejected under 35 USC § 112. These claims have been canceled without prejudice.

The Examiner acknowledges that the specification is enabling for methods of reconstituting an animal embryo by (i) transferring a donor nucleus from a diploid somatic cell into a first recipient oocyte; (ii) removing the nucleus from the first recipient oocyte; (iii) either activating a second recipient oocyte or enucleating a fertilized zygote; and (iv) transferring the nucleus from the first recipient oocyte into the preactivated second recipient oocyte or the enucleated fertilized zygote. However, the Examiner asserts that the specification does not reasonably provide enablement for methods to make any animal embryo or to use the resulting

embryo in methods to make any animal. Applicants note that reconstituted embryos can have other uses besides producing an animal. For example, page 3, lines 10-16; of the specification teach that the reconstituted animal embryos can be used to produce embryonic stem cells, embryonic germ cells, or other desired specialized or unspecialized cell type, e.g., neurons.

To support the lack of enablement rejection to make any animal embryo or to use the resulting embryo in methods to make any animal, the Examiner cites to a research article by Simerly et al. (Science 2003 300:297), as well as a commentary on Simerly's article by Vogel (Science 2003 300:225-227). The Examiner asserts that these articles establish the "art-recognized inability to generate a primate clone through methods of nuclear transfer". The Examiner concludes that since the instant specification fails to provide teachings to show that primate nuclear transfer using the claimed methods would result in pluripotent mammalian cells, it would require undue experimentation. Applicants respectfully disagree. In fact, Hwang et al. (Science. 2004 Mar 12; 303(5664):1669-74. Epub 2004 Feb 12; Attachment A) reported the derivation of a pluripotent embryonic stem (ES) cell line (SCNT-hES-1) from a cloned human blastocyst via somatic cell nuclear transfer. According to Steadman's Medical Dictionary (26th Edition, 1995), a blastocyst refers to the modified blastula stage of mammalian embryos. Thus, primate embryos and primate pluripotent mammalian cells derived therefrom have been produced via nuclear transfer.

In response to the Examiner's rejection that the specification does not reasonably provide enablement for methods to use the resulting embryo in methods to make any animal, Applicants attach hereto another technical comment on Simerly's article by Lanza et al. (Science 2002 301: 1482b; Attachment B). Lanza et al. comment that while Simerly et al. presented new data in their paper, they believe that Simerly et al. overstated their conclusions. In particular, Lanza et al. note that Simerly et al. transferred "only 33 rhesus embryos into 16 surrogates and concluded that reproductive cloning in primates may be unachievable". Lanza et al. further comment that "In our hands, it took dozens of embryos to generate Dolly, more than 150 embryos to generate the first cloned mouse pup and 586 embryos to establish the first two pregnancies in pigs." Lanza et al.'s comments reflect the art recognized fact that it can routinely require hundreds or thousands of embryo transfers to produce a viable cloned animal Also attached hereto is a table developed by Dr. Wilmut (one of the scientists that cloned Dolly) at the Roslin Institute (Somatic Cell Nuclear Transfer Cloning Efficiency" Paterson & Wilmut: www.roslin.ac.uk/public/webtablesGR.pdf; Attachment C). This table provides data on the

cloning efficiency of somatic cell nuclear transfer in seven different animals using multiple cell types and reveals that cloning efficiencies can be as low as 0.1%, which represents 1 live birth per 1000 embryos transferred to surrogate mothers. Interestingly, just last month, on August 3, 2005, the birth of the first cloned dogs was announced. Lee et al. (Nature. 2005 Aug 4; 436(7051):641; Attachment D) reported the cloning of two Afghan hounds by nuclear transfer from adult skin cells. Prior to Lee et al.'s success, failed attempts at cloning dogs were reported (Westhusin et al. 2001 J Reprod Fertil Suppl 57: 287-293; Attachment E) and many in the art doubted whether it could be achieved. However, Lee et al. were successful in producing two cloned dogs by transferring 1095 embryos into surrogate mothers. Furthermore, to date, at least 15 different animals have been cloned via nuclear transfer (see Table 1 below).

Table 1:

Animal Cloned	**Reference**
Mice	Wakayama et al. (1998). Nature, 394, 369-374;
Sheep	Schnieke et al. (1997), Science, 278, 2130-2133.
Pig	Polejaeva et al. (2000). Nature, 407, 86-90
Cattle	Kato et al. (1998). Science, 282, 2095- 2098
Goat	Keefer et al. (2000). Biology of Reproduction, 62, 218, Suppl 1.
Cat	Shin et al. (2002). Nature 415, 859
Rabbit	Chesné et al.(2002). Nature Biotechnology 20, 366-369.
Horse	Galli et al. (2003) Nature 424: 635
Rat	Zhou et al. (2003) Science 302: 1179
Mouflon	Loi et al. (2001)Nat Biotechnol. (10):962-4.
Deer	Press release
Mule	Woods et al. (2003) Science 301: 1063
Buffalo	Press release
Zebrafish	Lee et al. (2003) Nature Biotech 20: 795
Dog	Lee et al. (2005) Nature436(7051):641

Thus, Applicants believe that it would not require undue experimentation to practice the claimed invention. One skilled in the art recognizes that the successful cloning of animals is an inefficient process, which is taken into account by establishing an appropriate experimental design. Despite the fact that it could require hundreds or thousands of embryo transfers, this can routinely be accomplished by those skilled in the art in several weeks, and therefore doe not require "undue" experimentation.

It is respectfully believed that this application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is authorized to charge any fees due in connection with this response to Deposit Account No. 11-0980.

Respectfully submitted,
KING & SPALDING LLP

Stephanie DAdamo, Reg No 47,378
with express permission per
By Sherry M Knowles
Sherry M. Knowles, Esq.
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Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

REQUEST FOR EXTENSION OF TIME

The above-identified Applicant respectfully requests a two-month extension of time within which to file a response to the non-final Office Action dated April 13, 2005, to expire September 13, 2005.

Check No. 481328 in the amount of $225.00 is enclosed to cover the one-month extension of time fee. The Commissioner is hereby authorized to charge any underpayment of fees and to credit any overpayment to Deposit Account No. 11-0980.

Respectfully submitted,

Stefanie Adams Reg No 47,378
with express permission for
Sherry M. Knowles

Sherry M. Knowles, Esq.
Reg. No. 33,052

Date: September 13, 2005

KING & SPALDING LLP
191 Peachtree Street
45th Floor
Atlanta, GA 30303-1763

receipt of the items listed below.

Date Mailed September 13, 2005

Applicant : Keith Campbell

Appl. No. : 09/600,130

Filed : May 14, 2001

Title: Double Nuclear Transfer Method and Results Thereof

Papers Submitted: Response and Amendment to Non-Final Office Action (9pp.) Request for Extension of Time, Fee Transmittal, Check for $225.00, Certificate of Mailing, and Postcard

Attorney/Agent: Stephanie D. Adams

Docket No.: 10758.105003 REV1001

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ological grouping of the genotypes throughout the epidemic (Fig. 2) (table S2).

In tracing the molecular evolution of SARS-CoV in China, we observed that the epidemic started and ended with deletion events, together with a progressive slowing of the nonsynonymous mutation rates and a common genotype that predominated during the latter part of the epidemic. The mechanistic explanation for the selective adaptation and purification processes that led to such genomic evolutionary changes in SARS-CoV requires further work (*29*). Nonetheless, this study has provided valuable clues to aid further investigation of this remarkable evolutionary tale.

We have sequenced the complete S gene (GenBank accession number AY525636) from an oropharyngeal swab sample (sampling date, 22 December 2003) collected from the most recent index patient of the city of Guangzhou (onset date, 16 December 2003; hospitalized 20 December 2003; www.wpro.who.int/sars/docs/pressreleases/pr_27122003.asp). Phylogenetic analysis of this S gene sequence with those from the human SARS-CoV and palm civet SARS-like coronavirus indicated that this most recent case of SARS-CoV is much closer to the palm civet SARS-like coronavirus than to any human SARS-CoV detected in the previous epidemic (fig. S7 and table S4). Because it is evidently different from the recent laboratory infections in Singapore (www.who.int/csr/don/2003_09_24/en) and Taiwan (www.who.int/mediacentre/releases/2003/np26/en), it strengthens the argument for animal origin of the human SARS epidemic.

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17. Centers for Disease Control and Prevention, *Morb. Mortal. Wkly. Rep.* 52, 241 (2003).
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19. SARS-like coronaviruses were isolated from palm civets farmed domestically in Hubei Province, China, by Hu *et al.* at the Wuhan Institute of Virology, Chinese Academy of Sciences. Partial genome sequencing revealed an 82-nt deletion within the Orf8 region, which is identical to that found in human SARS-CoV isolates from the early patients of Zhongshan, Guangdong Province, China. Contamination can be ruled out because no human SARS-CoV isolate with the 82-nt deletion has ever been found in that institute or has been isolated in that region of China.
20. The SARS-CoV sequence with the 415-nt deletion (CUHK-LC2, CUHK-LC3, CUHK-LC4, and CUHK-LC5) was obtained from two SARS patients whose disease was linked to a late cluster of SARS cases in Hong Kong. Both patients had disease onset in mid-May 2003. The CUHK-LC2 sequence was initially obtained from the culture isolate of a throat wash specimen of an infected hospital health care worker and was later confirmed from the same specimen directly. CUHK-LC3, CUHK-LC4, and CUHK-LC5 were obtained from three different nasal swab specimens both directly and from the culture supernatants of an elderly patient who acquired SARS in the same hospital.
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30. See SOM Text at *Science* Online for acknowledgments.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1092002/DC1
Materials and Methods
SOM Text
References and Notes
Figs. S1 to S7
Tables S1 to S4

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Evidence of a Pluripotent Human Embryonic Stem Cell Line Derived from a Cloned Blastocyst

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Somatic cell nuclear transfer (SCNT) technology has recently been used to generate animals with a common genetic composition. In this study, we report the derivation of a pluripotent embryonic stem (ES) cell line (SCNT-hES-1) from a cloned human blastocyst. The SCNT-hES-1 cells displayed typical ES cell morphology and cell surface markers and were capable of differentiating into embryoid bodies in vitro and of forming teratomas in vivo containing cell derivatives from all three embryonic germ layers in severe combined immunodeficient mice. After continuous proliferation for more than 70 passages, SCNT-hES-1 cells maintained normal karyotypes and were genetically identical to the somatic nuclear donor cells. Although we cannot completely exclude the possibility that the cells had a parthenogenetic origin, imprinting analyses support a SCNT origin of the derived human ES cells.

The isolation of pluripotent human embryonic stem (ES) cells (*1*) and breakthroughs in somatic cell nuclear transfer (SCNT) in mammals (*2*) have raised the possibility of performing human SCNT to generate potentially unlimited sources of undifferentiated cells for use in research, with potential applications in tissue repair and transplantation medicine. This concept, known as "therapeutic cloning," refers to the transfer of the nucleus of a somatic cell into an enucleated donor oocyte (*3*). In theory, the oocyte's cytoplasm would reprogram the transferred nucleus by silencing all the somatic cell genes and activating the embryonic ones. ES cells would be isolated from the inner cell mass (ICM) of the cloned preimplantation embryo. When applied in a therapeutic setting, these cells would carry the nuclear genome of the patient; therefore, it is proposed that after directed cell differentiation, the cells could be transplanted without immune rejection to treat degenerative disorders such as diabetes, osteoarthritis, and Parkinson's disease

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(among others). Previous reports have described the generation of bovine ES-like cells (*4*) and mouse ES cells from the ICMs of cloned blastocysts (*5–7*) and the development of cloned human embryos to the 8- to 10-cell stage (*8*, *9*). Here we describe evidence of the derivation of human ES cells after SCNT (*10*).

Fresh oocytes and cumulus cells were donated by healthy women for the express purpose of SCNT stem cell derivation for therapeutic cloning research and its applications. Before beginning any experiments, we obtained approval for this study from the Institutional Review Board on Human Subjects Research and Ethics Committees (Hanyang University Hospital, Seoul, Korea). Donors were fully aware of the scope of our study and signed an informed consent form (a summary of the informed consent form is available in the supporting online text); donors voluntarily donated oocytes and cumulus cells (including DNA) for therapeutic cloning research and its applications only, not for reproductive cloning; and there was no financial payment. A total of 242 oocytes were obtained from 16 volunteers (there were one or two donors for each trial) after ovarian stimulation: 176 metaphase II (MII) oocytes were used directly for SCNT, whereas the remaining 66 oocytes were allowed to mature to the MII stage before use in SCNT. Autologous SCNT was performed; that is, the donor's own cumulus cell, isolated from the cumulus-oocyte complex (COC), was transferred back into the donor's own enucleated oocyte. Before enucleation, the oocytes were matured in vitro in G1.2 medium (Vitro Life, Goteborg, Sweden) for 1 to 2 hours. Enucleation, SCNT, and electrical fusion were performed as described (*11*). To directly confirm that the oocyte's DNA was removed during enucleation, we imaged the extruded DNA MII spindle complex from every oocyte with Hoechst 33342 fluorescent DNA dye (Fig. 1, A and B; arrows).

Without any report of an efficient protocol for human SCNT, several critical steps had to be optimized (*2*), including reprogramming time, activation method, and in vitro culture conditions. Reprogramming time, or the lapse of time between cell fusion and egg activation, returns the gene expression of the somatic cell to that needed for appropriate embryonic development. Initially, we investigated the effect of simultaneous fusion and activation, as used for porcine SCNT (*12*, *13*), but observed low fusion and cleavage rates, with no blastocyst development. Instead, we adapted the bovine SCNT procedure of waiting a few hours between fusion and activation. By allowing 2 hours for reprogramming, we were able to develop ~25% of the embryos to the blastocyst stage.

Because sperm-mediated activation is absent in SCNT, an artificial stimulus is needed to initiate development. Various chemical, physical, and mechanical agents induce parthenogenetic development in mice (*14*), but human data are limited. Oocyte activation using the calcium ionophore A23187 (calcimycin) or ionomycin and the protein synthesis inhibitor puromycin induces parthenogenetic development of human oocytes at different efficiencies (*15*). We found that incubation in 10 μM A23187 for 5 min, followed by incubation with 2.0 mM 6-dimethylaminopurine (DMAP) for 4 hours, gave efficient chemical activation for human SCNT eggs. Other investigators have reported encouraging results in overcoming inefficiencies in embryo culture by supplementing the culture with different energy substrates (*16*). Furthermore, the recent development of serum-free sequential media has led to considerable improvement in the rate of clinical pregnancies produced by in vitro fertilization (IVF) (*17*). In this study, human modified synthetic oviductal fluid with amino acids (hmSOFaa) was prepared by supplementing mSOFaa (*18*) with human serum albumin (10 mg/ml) and fructose (1.5 mM) instead of bovine serum albumin (8 mg/ml) and glucose (1.5 mM). The replacement of glucose with fructose improves the developmental competence of bovine SCNT embryos (*11*, *19*). Culture of human SCNT-derived embryos in G1.2 medium for the first 48 hours followed by hmSOFaa medium produced more blastocysts, as compared to culture in G1.2 medium for the first 48 hours followed by culture in G1.2 medium or in continuous hmSOFaa medium (Table 1). Cibelli *et al.* (*8*) reported that the treatment of human oocytes with 5 μM calcium ionomycin followed by 2 mM DMAP in G1.2 culture medium triggered pronucleus formation, embryonic cleavage, and the formation of a blastocoelic cavity in human parthenotes. However, they did not obtain human SCNT blastocysts when their protocol was applied to SCNT embryos. Limitations in oocyte supply precluded full optimization of all the parameters for human SCNT; nonetheless, the protocol described here produced cloned blastocysts at rates of 19 to 29% (as a percentage of oocytes used) and was comparable to those produced by established SCNT methods in cattle (~25%) (*11*) and pigs (~26%) (*12*, *13*).

A total of 30 SCNT-derived blastocysts were cultured, 20 ICMs were isolated by immunosurgical removal of the trophoblast, and one ES cell line (SCNT-hES-1) was derived. The resulting SCNT-hES-1 cells had a high nucleus-to-cytoplasm ratio and prominent nucleoli. The cell colonies displayed similar morphology to that reported previously for hES cells derived after IVF (Fig. 1, C to E). When cultured in defined medium conditioned for neural cell differentiation (*20*), SCNT-hES-1 cells differentiated into nestin-positive cells, an indication of primitive neuroectoderm differentiation (Fig. 1F). The SCNT-hES-1 cell line was mechanically passaged by dissociation every 5 to 7 days and successfully maintained its undifferentiated mor-

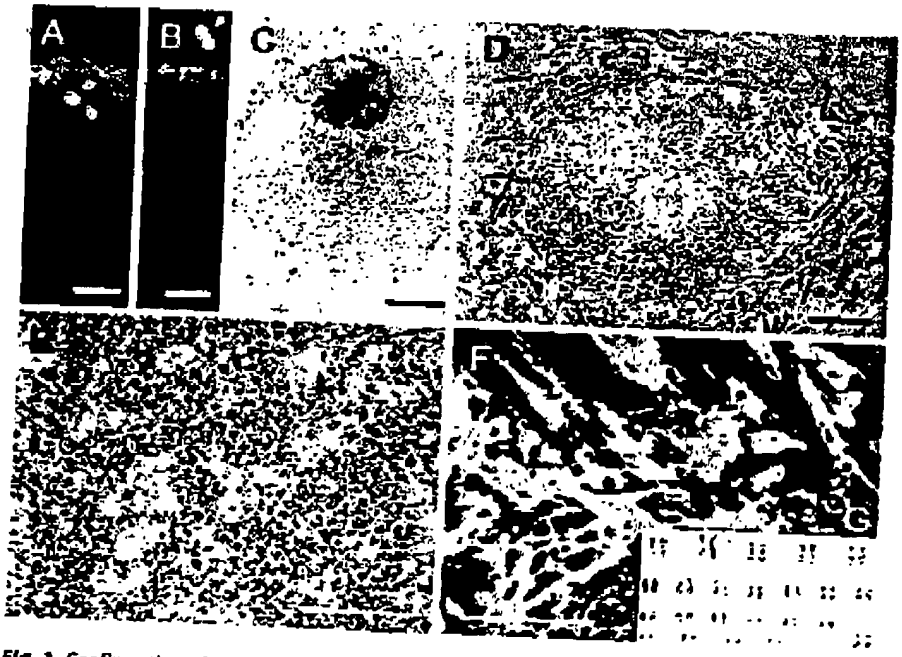

Fig. 1. Confirmation of enucleation, photographs of human SCNT ES cells and their differentiated progeny, and karyotype analysis. (A and B) Images (×200) of extruded DNA MII spindle complexes (arrows) from an oocyte before (A) and after (B) enucleation. (C to E) Bright-field [(C), ×100] and phase contrast [(D), ×100] micrographs and higher magnification image [(E), ×200] of a colony of SCNT-hES-1 cells. Immunofluorescence staining for nestin [(F), ×200] and G-banded karyotyping (G) in SCNT-hES-1 cells are shown. Scale bars, 20 μm in (A) and (B) and 100 μm in (C) to (F).

phology after continuous proliferation for >70 passages, while still maintaining a normal female (XX) karyotype (Fig. 1G) (*21*). Furthermore, the SCNT-hES-1 cells expressed ES cell markers such as alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Oct-4, but not SSEA-1 (Fig. 2). As previously described in monkey (*22*) and human ES cells (*1*, *23*, *24*) and in mouse SCNT-ES cells (*6*), SCNT-hES-1 cells did not respond to exogenous leukaemia inhibitory factor, suggesting that a pluripotent state is maintained by a gp130-independent pathway. The pluripotency of SCNT-hES-1 cells was investigated in vitro (fig. S1) and in vivo (Fig. 3). Clumps of the cells were cultured in vitro in suspension to form embryoid bodies. The resulting embryoid bodies were stained with three dermal markers and were found to differentiate into a variety of cell types, including derivatives of endoderm, mesoderm, and ectoderm (fig. S1). When undifferentiated SCNT-hES-1 cells were injected into the testes of severe combined immunodeficient (SCID) mice, teratomas were obtained 6 to 7 weeks after injection. The resulting teratomas contained tissue representative of all three germ layers. Differentiated tissues seen in Fig. 3 include neuroepithelial rosset, pigmented retinal epithelium, smooth muscle, bone, cartilage, connective tissues, and glandular epithelium. The DNA fingerprinting analysis with human short tandem repeat (STR) markers indicates that the cell line originated from the cloned blastocysts reconstructed from the donor cells, not from parthenogenetic activation (Fig. 4, A to C). The statistical probability that the cells may have derived from an unrelated donor is 8.8×10^{-16}. Reverse transcription polymerase chain reaction (RT-PCR) amplification for paternally expressed (*hSNRPN* and *ARHI*) and maternally expressed (*UBE3A* and *H19*) genes further confirmed that the cell line originated from the donor cells (Fig. 4D).

Simerly *et al.* (*26*) recently reported defective mitotic spindles after SCNT in nonhuman primate embryos, perhaps resulting from the depletion of microtubule motor and centrosome proteins lost to the meiotic spindle after enucleation. In this study, Fig. 1G demonstrates that SCNT-hES-1 cells have the normal karyotype. We speculate that SCNT blastocysts from which ES cell lines were not derived might have been aneuploid. However, it is important to note that our investigations differ from those of Simerly *et al.* in a few ways: Media and protocols for in vitro development were optimized for human oocytes and embryos, whereas the protocols for nonhuman primate studies are extrapolated from clinical procedures; the enucleation method here differs, because we squeeze the MII oocyte so that the DNA spindle complex is extruded through a small hole in the zona pellucida, instead of aspirating the DNA spindle complex with a glass pipette as others have described (*27*); and the DNA spindle complex is extruded shortly after the appearance of the first polar body, so that it may even be at the prometaphase II stage.

In this report, we provide three lines of evidence supporting the nuclear transfer origins of the SCNT-hES-1 cell line: (i) DNA extraction was verified for each of the 242 enucleated oocytes (Fig. 1, A and B; arrows); (ii) DNA fingerprinting showed heterozy-

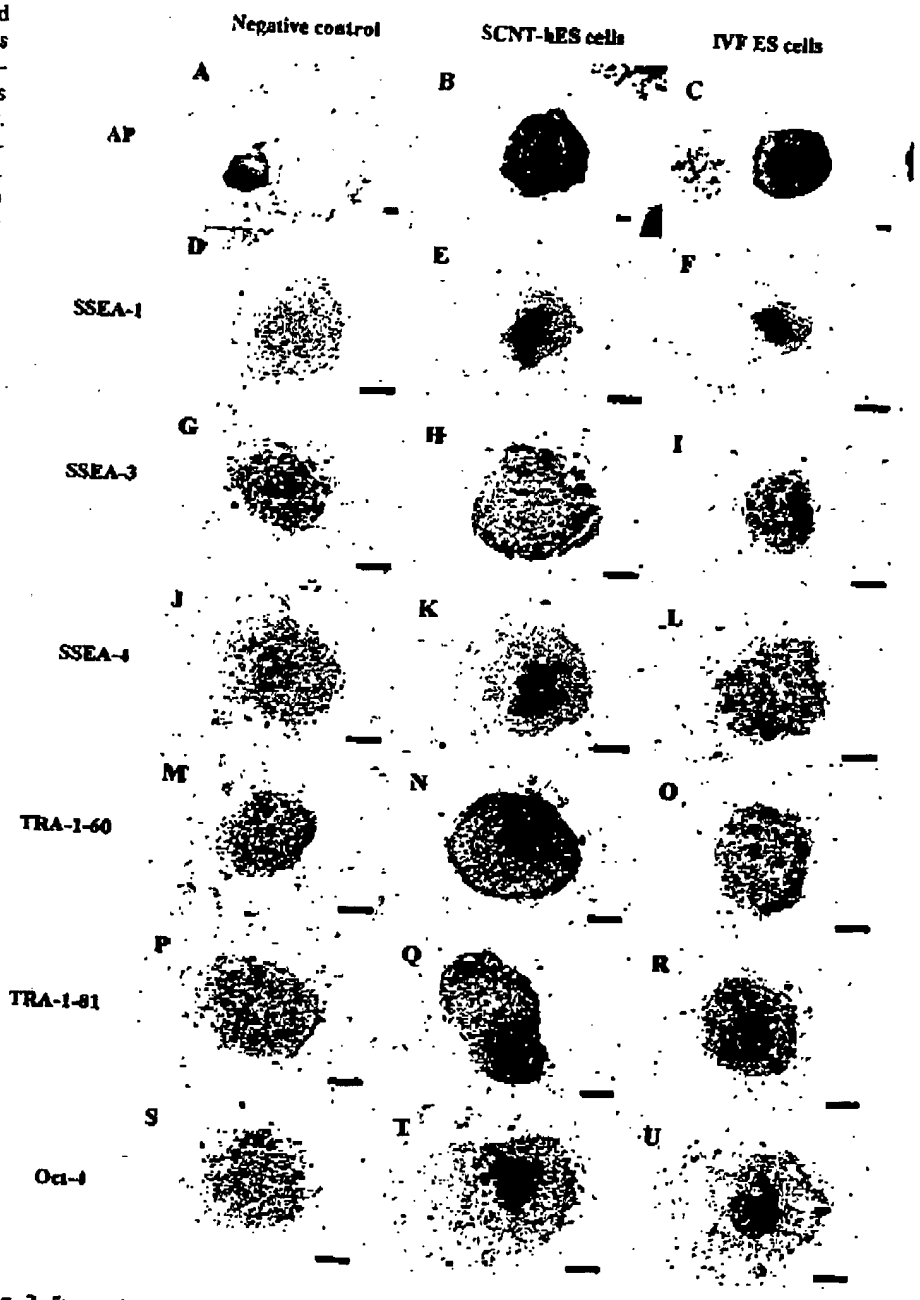

Fig. 2. Expression of characteristic cell surface markers in human SCNT ES cells. SCNT-hES-1 cells expressed cell surface markers, including alkaline phosphatase (B), SSEA-3 (H), SSEA-4 (K), TRA-1-60 (N), TRA-1-81 (Q), and Oct-4 (T), but not SSEA-1 (E). The differentiated SCNT-hES-1 cells were not stained with alkaline phosphatase (A). The IVF-derived human ES cells (Miz-hES) were used for comparison and also expressed alkaline phosphatase (C), SSEA-3 (I), SSEA-4 (L), TRA-1-60 (O), TRA-1-81 (R), and Oct-4 (U), but not SSEA-1 (F). Negative controls not treated with first antibodies are shown (D, G, J, M, P, and S). Magnification in (A) to (U), ×40. Scale bars, 100 μm.

gous, not homozygous, chromosomes (Fig. 4, A to C); and (iii) RT-PCR showed biparental, and not uniparental, expression of imprinted genes (Fig. 4D). Although the Cyno-1 parthenogenetic cells retained their strictly maternal imprints, that evidence came from a single monkey cell line. Given the aberrant expression of imprinted genes after murine SCNT (*28*), perhaps the SCNT-hES-1 cells' biparental expression of imprinted genes might have been influenced by SCNT or subsequent culture. Heterologous along with autologous SCNT will provide more definitive molecular evidence. Although overwhelming ethical constraints preclude any reproductive cloning attempts, complementary investigations in nonhuman primates might provide additional and confirmatory information. Consequently, although we cannot exclude the possibility of a parthenogenetic origin, the studies reported here support the conclusion that the SCNT-hES-1 cell line originated from the donor's diploid somatic cumulus cell after SCNT.

In order to successfully derive immunocompatible human ES cells from a living donor, a reliable and efficient method for producing cloned embryos and ES isolation must be developed. Thomson *et al.* (*1*), Reubinoff *et al.* (*23*), and Lanzendorf *et al.* (*29*) produced human ES cell lines at high efficiency. Briefly, five ES cell lines were derived from a total of 14 ICMs, two ES cell lines were derived from four ICMs, and three ES cell lines were derived from 18 ICMS, respectively. In our study, one SCNT-hES cell line was derived from 20 ICMs. It remains to be determined whether this low efficiency is due to faulty reprogramming of the somatic cells or to subtle variations in our experimental procedures. We cannot rule out the possibility that the genetic background of the cell donor had an impact on the overall efficiency of the procedure. Further improvements in SCNT protocols and in vitro culture

Fig. 3. Teratomas formed by human SCNT ES cells in the testes of SCID mice at 12 weeks after injection. Neuroepithelial rosset (A), pigmented retinal epithelium (B), osteoid island showing bony differentiation (C), cartilage (D), and glandular epithelium with smooth muscle and connective tissues (E). Magnification in (A) to (D), ×200; in (E), ×100. Scale bar, 100 μm.

Table 1. Conditions for human SCNT.

Experiment	Activation condition*	Reprogramming time (hours)	1st step medium†	2nd step medium	No. of oocytes	No. (%) of cloned embryos developed to		
						Two-cell	Compacted morula	Blastocyst
1st set	10 μM ionophore 6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	4 (25)	4 (25)
	10 μM ionophore 6-DMAP	4	G 1.2	hmSOFaa	16	15 (94)	1 (6)	0
	10 μM ionophore 6-DMAP	6	G 1.2	hmSOFaa	16	15 (94)	1 (6)	1 (6)
	10 μM ionophore 6-DMAP	20	G 1.2	hmSOFaa	16	9 (56)	1 (6)	0
2nd set	10 μM ionophore 6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	5 (31)	3 (19)
	5 μM ionophore 6-DMAP	2	G 1.2	hmSOFaa	16	11 (69)	0	0
	10 μM ionomycin 6-DMAP	2	G 1.2	hmSOFaa	16	12 (75)	0	0
	5 μM ionomycin 6-DMAP	2	G 1.2	hmSOFaa	16	9 (56)	0	0
3rd set	10 μM ionophore 6-DMAP	2	G 1.2	hmSOFaa	16	16 (100)	4 (25)	3 (19)
	10 μM ionophore 6-DMAP	2	G 1.2	G 2.2	16	16 (100)	0	0
	10 μM ionophore 6-DMAP	2	Continuous hmSOFaa		16	16 (100)	0	0
4th set	10 μM ionophore 6-DMAP	2	G 1.2	hmSOFaa	66	62 (93)	24 (36)	19 (29)

*Fused donor oocytes and somatic cells were activated in either calcium ionophore A23187 (5 or 10 μM) or ionomycin (5 or 10 μM) for 5 min, followed by 2 mM 6-DMAP treatment for 4 hours. †Oocytes were incubated in the first medium for 48 hours.

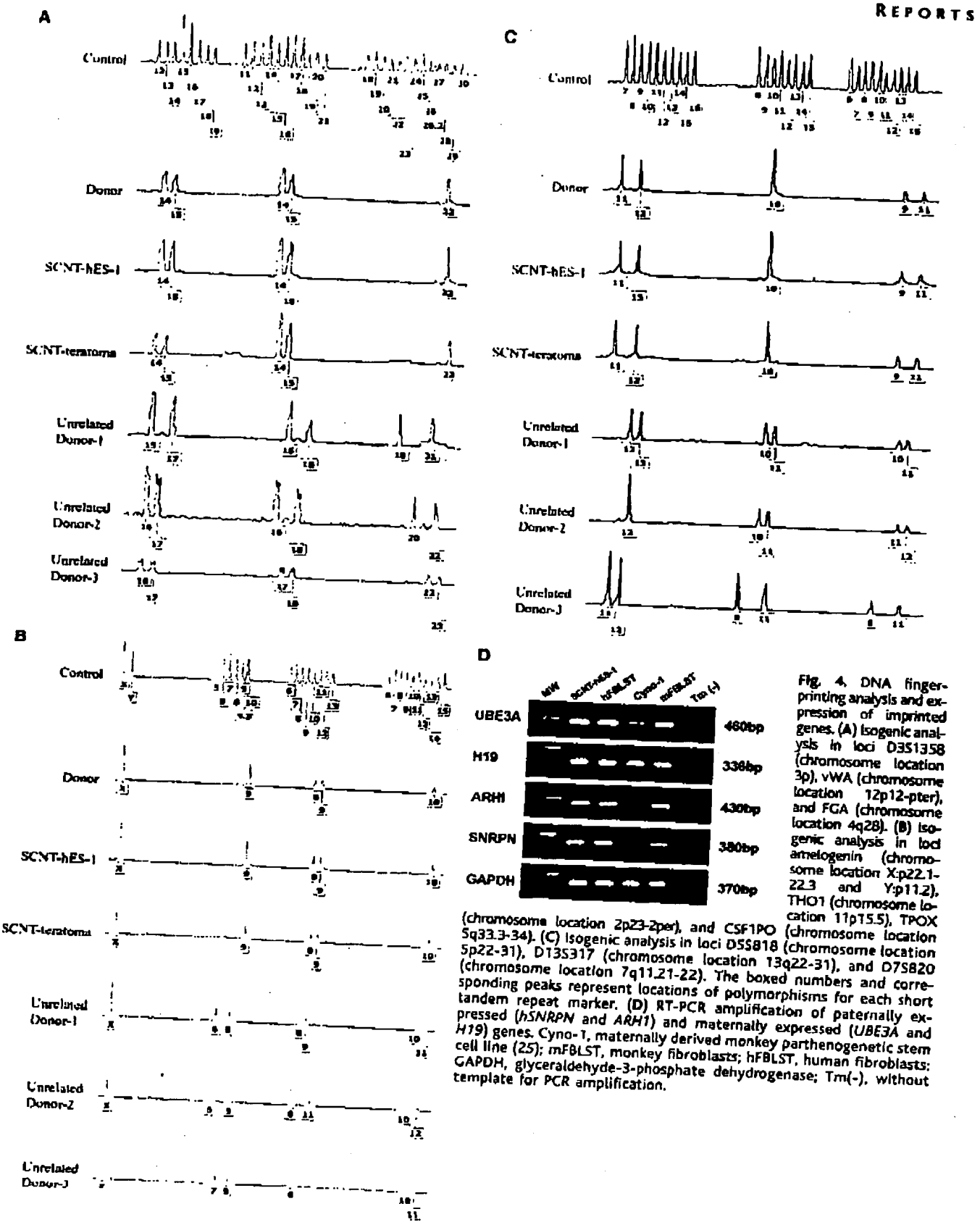

Fig. 4. DNA fingerprinting analysis and expression of imprinted genes. (A) Isogenic analysis in loci D3S1358 (chromosome location 3p), vWA (chromosome location 12p12-pter), and FGA (chromosome location 4q28). (B) Isogenic analysis in loci amelogenin (chromosome location X:p22.1-22.3 and Y:p11.2), THO1 (chromosome location 11p15.5), TPOX (chromosome location 2p23-2per), and CSF1PO (chromosome location 5q33.3-34). (C) Isogenic analysis in loci D5S818 (chromosome location 5p22-31), D13S317 (chromosome location 13q22-31), and D7S820 (chromosome location 7q11.21-22). The boxed numbers and corresponding peaks represent locations of polymorphisms for each short tandem repeat marker. (D) RT-PCR amplification of paternally expressed (*hSNRPN* and *ARH1*) and maternally expressed (*UBE3A* and *H19*) genes. Cyno-1, maternally derived monkey parthenogenetic stem cell line (*25*); mFBLST, monkey fibroblasts; hFBLST, human fibroblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tm(-), without template for PCR amplification.

systems are needed before contemplating the use of this technique for cell therapy. In addition, the mechanisms governing the differentiation of human tissues must be elucidated in order to produce tissue-specific cell populations from undifferentiated ES cells. This study shows the feasibility of generating human ES cells from a somatic cell isolated from a living person.

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Force-Clamp Spectroscopy Monitors the Folding Trajectory of a Single Protein

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We used force-clamp atomic force microscopy to measure the end-to-end length of the small protein ubiquitin during its folding reaction at the single-molecule level. Ubiquitin was first unfolded and extended at a high force, then the stretching force was quenched and protein folding was observed. The folding trajectories were continuous and marked by several distinct stages. The time taken to fold was dependent on the contour length of the unfolded protein and the stretching force applied during folding. The folding collapse was marked by large fluctuations in the end-to-end length of the protein, but these fluctuations vanished upon the final folding contraction. These direct observations of the complete folding trajectory of a protein provide a benchmark to determine the physical basis of the folding reaction.

Resolving the folding pathway of a protein remains a challenge in biology (*1*–*9*). Here, we demonstrate a method by which the entire folding trajectory of a single protein can be recorded as a function of time. We used single-molecule atomic force microscopy techniques (*10*, *11*) in the force-clamp mode (*12*, *13*) to apply a constant force to a single polyprotein composed of nine repeats of the small protein ubiquitin (*13*–*16*). This resulted in the probabilistic unfolding of ubiquitin, which was observed as stepwise elongations of the protein in which each step corresponded to the unfolding of an individual protein module (*12*). We applied this technique to monitor the end-to-end length of a single ubiquitin polyprotein (*17*) during reversible unfolding-folding cycles. Our experimental approach is illustrated in Fig. 1. Figure 1A shows the changes in the length of a single ubiquitin polyprotein in response to the stretching force displayed in Fig. 1B. As shown, stretching the polyubiquitin chain at 120 pN triggers a series of unfolding events that appear as a staircase of 20-nm steps, marking the unfolding of the individual ubiquitins in the chain (Fig. 1A). After 4 s, the force was relaxed to 15 pN (Fig. 1B) (*18*), and we observed the protein spontaneously contract in stages until it reached its folded length (Fig. 1A). To confirm that the polyprotein had folded, we raised the stretching force back to 120 pN at 14 s (Fig. 1B) and observed the ubiquitin chain extend in steps of 20 nm back to its fully unfolded length (Fig. 1A). Hence, the spontaneous contraction of the protein observed upon reducing the force from 120 pN down to 15 pN corresponds to the folding trajectory of the mechanically unfolded ubiquitin.

We observed and analyzed 81 folding events similar to those shown in Fig. 1. Two typical folding trajectories for mechanically unfolded polyubiquitin molecules are shown in Fig. 2. Most of the folding trajectories are qualitatively similar, following a continuous convex time course marked by abrupt changes in slope. However, we have never observed identical sets of trajectories, indicating the existence of multiple folding pathways for ubiquitin. To simplify the analysis of the folding trajectories, we divided their time course, roughly, into four distinct stages marked by abrupt changes in the slope of the collapse (Fig. 2). As an example, we analyze the recording shown in Fig. 2A. The first stage (1 in Fig. 2A and inset) is fast, lasting ~10 ms, which is slower than the time it takes the force to reach its set point (~3 ms in this experiment). The collapse rate for this stage (cr_1 = 2135 nm/s) is within the range but clearly slower than the maximum rate of change, or slew rate (*sr*), of the feedback during this experiment (measured at *sr* = −8300 nm/s, after the molecule detached from the cantilever). This stage is likely to correspond to the elastic recoil of the unfolded polypeptide chain adjusting its length to the step change in the pulling force. This stage is always fast and is clearly marked in

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TECHNICAL COMMENT

Comment on "Molecular Correlates of Primate Nuclear Transfer Failures"

We believe that Simerly *et al.* (*1*) have reported important new data but have overstated their conclusions. Their preliminary observations highlight the importance of egg quality—centrosome and spindle competence, specifically—in nuclear transfer (NT) procedures. However, the data are scant and their interpretation is compromised by the multiplicity of methods used. In our own experience, multiple factors including donor and recipient cell cycle stage and oocyte age can influence the integrity of the spindle complex. Simerly *et al.* buttressed their claim that primates are unique in the specific absence of NuMA (nuclear-mitotic apparatus protein) or HSET based only on immunofluorescence. However, one cannot conclude from these data that the oocyte was depleted of these proteins—only that they were not detected where expected using the techniques described. The occurrence of abnormal spindles has long plagued NT in most species, and few comparable immunocytochemical studies have been reported to support the conclusion that failed spindle complexes in other species would not lack those markers as well. More comprehensive investigations of NT-induced centrosome-spindle perturbations will reveal the true impact of these manipulations on the efficiency of NT on a species-by-species basis.

Simerly *et al.* transferred only 33 rhesus embryos into 16 surrogates and concluded that reproductive cloning in primates may be unachievable. In a related news article (*2*), the senior author of (*1*) stated that it is almost as if someone "drew a sharp line between old-world primates—including people—and other animals, saying, 'I'll let you clone cattle, mice, sheep, even rabbits and cats, but monkeys and humans require something more.' " In our own hands, it took dozens of embryos to generate Dolly (*3*), more than 150 embryos to generate the first cloned mouse pup (*4*),and 586 embryos to establish the first two pregnancies in pigs (*5*). Hundreds of other studies have ended with no pregnancies at all. We suggest that given the potential importance of nuclear transfer techniques in human cell therapies, conclusions regarding the efficiency of human NT extrapolated from animal data should be tempered with abundant caution.

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Somati cll Nuclear Transfer (Cloning) i .ciency

The overall efficiency of cloning is typically between 0 and 3 % (number of live offspring as a percentage of the number of nuclear transfer embryos), irrespective of the species, the donor cell type or technique. However, this is the cumulative result of losses at each stage of the cloning process. The following tables provide a summary of the data for each published report on somatic cell (fetal to adult cells) nuclear transfer to date. Where the appropriate data was made available, the table provides cloning efficiency for each major stage of the process:

% Embryos formed from NT Oocytes: % of morula and/or blastocysts formed from the number of NT (nuclear transfer) oocytes created. The number of NT oocytes is the number of oocytes that were enucleated and injected or fused with donor nuclei. In some reports, only the number of oocytes cultured, or surviving activation or injection is given and the figures are denoted by * with a corresponding footnote.

No. Implantation Sites: This column refers to mice only, and shows how many fetuses implanted into the uterus but were subsequently reabsorbed.

No. Pregnant (no. of recipients): This column is provided for each livestock species. The time of pregnancy confirmation is species dependent. (no. of recipients) refers to the total number of recipients to which embryos were transferred.

No. Pregnancies Lost: Refers to the number of recipients that had lost their fetuses at various stages throughout gestation.

Live Offspring (% embryos transferred): Refers to the number of live offspring born and is also given as a % of the total number of embryos transferred (normally morula or blastocyts, however some studies transfer 2,4,and 8 cell).

No. Survived: Refers to the number of live offspring that survived.

CE: Cloning Efficiency is the number of live offspring expressed as a % of the total number of NT oocytes (this is generally the number of oocytes injected or fused but depending on the data this may refer to, for example, the number of oocytes surviving injection or the number cultured. Furthermore it should be noted that in many studies, not all of the embryos that developed are transferred to recipient females).

Ref: The subscript refers to the reference bibliography provided at the bottom of this article.

	Donor Cell Age	Donor Cell	% Embryos formed from NT Oocytes	No. Implantation Sites	Live Offspring (% embryos transferred)	No. Survived	CE Ref
	Adult	Cumulus			31 (2.2%)	22	1.3% 1
					86 (2.3%)	80	1.1 % 2
			42.1%		7 (23.3%)	4	5.8% 3
					3 (0.7%)	0	? 4
			46.0%		6 (2.0%)	?	0.9% 5
					7 (2.7%)	?	2.3% 5
			52.8%		9 (5.3%)	?	2.8% 5
		Fibroblasts			3 (1.1%)	1	0.4% 6
			21.8%*	134	5 (1.7%)	5	0.3% 7
	Newborn	Sertoli	23.6%**	235	16 (3.6%)	11	0.9% 8
	Fetal	Fibroblasts	16.4%	73	5 (1.1%)	2	0.2% 9
		Gonad	55.1%		6 (2.7%)	5	1.5% 2
		Neural cells	29.7%		12 (10.3%)	11	3.1% 10

No. of NT oocytes except * no. of surviving oocytes, ** no. oocytes cultured

	Donor Cell Age	Donor Cell	% Embryos formed from NT Oocytes	No. Pregnant (no. recipients)	No. Pregnancies Lost	Live Offspring (% embryos transferred)	No survived	CE Ref
	Adult	Cumulus	39.4%	3 (3)	0	5 (83.3%)	2	5% [11]
				6 (34)	3	3 (7.3%)	3	0.9% [12]
		Oviduct	39.3%	2 (2)	0	3 (75%)	2	2% [11]
				4 (14)	2	2 (11.8%)	2	0.8 [12]
		Uterine		3 (7)	2	2 (14.2%)	0	2% [12]
		Granulosa	69.4%			10 (10%)	10	1.8% [13]
						2 (2.7%)	1	0.5% [14]
			16.0%	31 (91)	23	9	8	0.7% [15]
		Mammary Gland	16.1%	2 (2)	1	1 (25%)	1	0.4% [16]
			16.8%	4 (31)	2	2 (4.4%)	2	0.7% [17]
		Muscle	14.2%	8 (20)	4	4 (15.3%)	1	0.8% [18]
		Fibroblasts	53.3%	5 (12)	4	1 (6.25%)	0	1.1% [16]
				1 (5)	0	1 (16.7%)	0	0.6% [19]
			11.9%	15 (36)	9	6 (7.4%)	4	0.5% [20]
			16.5%	6 (11)	5	1 (3.8%)	1	0.3% [21]
				24 (48)	12	12 (20.3%)	4	2.8% [12]
			31.1%	6 (37)	4	2 (4.6%)	1	0.8% [17]
			?	45 (133)	36	9	6	? [22]

	Donor Cell Age	Donor Cell	% Embryos formed from NT Oocytes	No. Pregnant (no. recipients)	No. Pregnancies Lost	Live Offspring (% embryos transferred)	No survived	CE Ref
	New-born	Fibroblasts		5 (13)	3	2 (12.5%)	2	1.1% 12
		Liver		4 (8)	2	2 (20%)	1	1.3% 12
		Testis		1 (1)	1	0	/	0% 12
	Fetal	Fibroblasts	4.6%	17 (32)	11	6 (7.6%)	6	0.3% 23
			22.7%	8 (12)	7	2 (8.7%)	1	0.4% 24
			11.7%	5 (12)	5	0	/	0% 21
				2 (6)	1	1 (14.3%)	1	3.8% 12
			27.5%	52 (115)	33	25 (11.3%)	11	0.9% 25
				11 (40)	5	6	5	? 22
				110 (247)	80	30 (6.0%)	24	? 26
		Germ Cells	22.7%	8 (17)	7	1 (3.1%)	0	0.2% 27
		Liver		1 (3)	1	0	/	0% 12
	Fetal Adult	Somatic		535 (2170)	432	106	82	? 28

	Donor Cell Age	Donor Cell	% Embryos formed from NT Oocytes	No. Pregnant (no. recipients)	No. Pregnancies Lost	Live Offspring (% embryos transferred)	No. survived	CE
								Ref
	Adult	Fibroblasts		2 (5)	1	2 (0.9%)	2	0.9% [9]
		Granulosa		2 (10)	1	5 (0.8%)	5	0.1% [9]
	New-born	Fibroblasts	9.5%	1 (5)	0	4 (0.9%)	2	? [9]
	Fetal	Fibroblasts				1 (0.9%)	1	0.2% [12]
				3 (9)	2	1	1	0.1% [33]
				5 (10)	3	2 (0.3%)	1	0.2% [34]
		Somatic				4 (0.7%)	4	0.5% [35]
	Adult	Mammary Gland	10.5%	1 (13)	0	1 (3.4%)	1	0.4% [36]
		Granulosa	30.4%	2 (4)	1	1	1	4.3% [17]
			11.9%	31		6 (6.7%)	1	0.6% [38]
	Fetal	Fibroblasts	27.3%	5 (16)	2	3 (7.5%)	2	1.7% [36]
			13.6%	11 (24)	2	7 (10.4%)	5	1.4% [39]
				39 (78)	31	4 (3.3%)	0	? [40]
			19.2%	17 (42)	3	14 (17.5%)	3	3.4% [41]
	Adult	Granulosa		4 (9)		7 (7.3%)	6	? [42]
		Cumulus		4 (8)	0	7 (7.7%)	6	7.2% [43]
	Fetal	Fibroblasts		2 (29)	0	3 (1.3%)	1	0.7% [44]
				1 (6)	0	2 (3.7%)	1	2.1% [45]
				5 (13)	0	6 (6.2%)	3	1.6% [46]
				5 (23)	0	5 (2.2%)	5	0.8% [46]
			32.1%	6 (15)	3	5 (13.2%)	5	3.1% [47]
		Somatic				3 (2.7%)	3	1.1% [48]
	Adult	Cumulus or Fibroblast		2 (8)	1	1 (1.1%)	1	? [49]
	Adult	Cumulus		10 (64)	6	6 (0.6%)	4	0.3% [50]

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BRIEF COMMUNICATIONS

Dogs cloned from adult somatic cells

Two Afghan pups could help to unravel the genetics behind the assorted traits of other canine breeds.

Several mammals — including sheep, mice, cows, goats, pigs, rabbits, cats[1], a mule[2], a horse[3] and a litter of three rats[4] — have been cloned by transfer of a nucleus from a somatic cell into an egg cell (oocyte) that has had its nucleus removed. This technology has not so far been successful in dogs because of the difficulty of maturing canine oocytes *in vitro*. Here we describe the cloning of two Afghan hounds by nuclear transfer from adult skin cells into oocytes that had matured *in vivo*. Together with detailed sequence information generated by the canine-genome project[5,6], the ability to clone dogs by somatic-cell nuclear transfer should help to determine genetic and environmental contributions to the diverse biological and behavioural traits associated with the many different canine breeds[7,8].

Successful somatic-cell nuclear transfer (SCNT) depends on the quality, availability and maturation of the animal's unfertilized oocytes. Unlike other mammals, dogs ovulate at first meiotic prophase, and their oocytes mature for 2 to 3 days in the oviduct's distal regions. Previously, intra- and interspecific canine embryos have been constructed by canine SCNT into canine and bovine oocytes, respectively, but this did not result in viable offspring[9].

We collected oocytes matured *in vivo* at metaphase II about 72 hours after ovulation by flushing the oviducts. (For details of methods, see supplementary information.) Donor fibroblasts were obtained from an ear-skin biopsy of a male Afghan hound and cultured for two to five passages (in which fully grown cells are transferred to a new culture dish). For SCNT, the chromosomes of the unfertilized canine oocytes were removed by micromanipulation, and a single donor cell was transferred into each enucleated oocyte. The couplets were fused and only successfully fused couplets (75%) were activated. The activated oocytes were then transferred into the oviducts or uterine horns of recipient dogs at times appropriate to the embryos' developmental stages. We collected an average of 12 oocytes from each female, and a total of 1,095 reconstructed canine embryos were transferred into 123 recipients.

Three pregnancies were confirmed by ultrasound scans at 22 days' gestation in recipients after transfer of constructs. Pregnancy was established only after embryo transfer of very-early-stage nuclear-transfer constructs (that is less than 4 hours after oocyte activation). This transfer of early-stage embryos is a crucial factor in successful assisted reproductive technology for dogs. One fetus miscarried and two others were carried to term.

Figure 1 | Dog cloned by somatic-cell nuclear transfer. a, Snuppy, the first cloned dog, at 67 days after birth (right), with the three-year-old male Afghan hound (left) whose somatic skin cells were used to clone him. Snuppy is genetically identical to the donor Afghan hound. b, Snuppy (left) was implanted as an early embryo into a surrogate mother, the yellow Labrador retriever on the right, and raised by her.

We named the first cloned dog Snuppy (for Seoul National University puppy); it is shown in Fig. 1a with the male Afghan fibroblast donor. Snuppy was delivered by caesarian section after 60 days (full term) from a yellow Labrador surrogate mother (Fig. 1b); his birth weight was 530 g. The second SCNT dog, NT-2, was carried by a mixed-breed surrogate, and was also delivered at 60 days, weighing 550 g (normal range for Afghans in a litter, 482–680 g). He experienced neonatal respiratory distress during the first week, seemed to recover, but died on day 22 as a result of aspiration pneumonia; no major anatomical anomalies were evident post mortem.

We tested whether the cloned dogs were genetically identical by microsatellite analysis of genomic DNA from the donor Afghan, the cloned dogs and the surrogates (see supplementary information). Analysis of eight canine-specific microsatellite loci confirmed that the cloned dogs were genetically identical to their donor dog. However, the efficiency of cloning is still very low (2 dogs from 123 recipients, or 1.6%) compared with the rates for cats[1] and horses[3].

In addition to the benefits that cloning technology may generally provide (the preserva- — once canine embryonic stem cells become available), this technology could become a useful research tool for studying the genetics of outcrossed populations.

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Potential for cloning dogs

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The aim of this study was to determine whether nuclear transplantation could be used to clone a dog using donor nucleus cells collected from an adult female. Fibroblasts obtained from skin biopsies were fused with enucleated bovine or canine oocytes. The resulting cloned embryos were cultured *in vitro* to monitor embryonic development. A proportion of the resulting embryos was transferred into surrogate bitches for development to term. When canine oocytes were used as recipient ova for canine fibroblasts, 23% of the resulting embryos cleaved at least once after culture *in vitro*. Five cloned embryos were transferred into three synchronized recipient bitches, but no pregnancies resulted. When bovine oocytes were used as recipients for canine fibroblasts, 38% cleaved to the two- to four-cell stage and 45% cleaved to the eight- to 16-cell stage. Forty-seven of these embryos were transferred into four recipient females, resulting in a single conceptus that ceased development at about day 20 of gestation. The desire for cloned dogs is considerable and will undoubtedly incite the development of successful methods for cloning companion animals. However, significant investment into additional research is required, especially in the areas of *in vitro* maturation of oocytes and control of the oestrous cycle of bitches.

Introduction

Cloned sheep, cattle and mice have been produced by nuclear transplantation using somatic cells obtained from adult animals (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Renard *et al.*, 1999; Wells *et al.*, 1999; Wakayama and Yanagimachi, 1999; Hill *et al.*, 2000). There is no evidence to suggest that cloning will be limited to only a few species and, in fact, most of the evidence collected indicates that cloning will be applicable to a wide variety of different animals. Cloning dogs and other pets has recently gained considerable public interest. This was highlighted in 1998 by the public announcement of the Missyplicity Project, a multimillion dollar research effort being carried out in our laboratory, which is targeted at developing technology for cloning dogs. A single private investor who wishes to remain anonymous is funding the Missyplicity Project, but the desire to clone dogs is shared by a large number of individuals. The reasons for wanting to clone dogs range from the desire to replicate the genotypes of superior service dogs to applications in endangered species. However, the greatest interest comes from individuals wanting to replicate the genotype of their own pet. Dogs represent a significant challenge compared with other species that have been cloned, simply because many of the basic mechanisms controlling reproduction are not yet well understood. Efficient and repeatable techniques for assisted reproduction in dogs including superovulation, synchronization of oestrus, embryo collection, embryo transfer and *in vitro* production of embryos, are not yet commonplace. The aim of this study was to determine whether techniques similar to those used for cloning livestock could be used to produce genetically identical dogs.

Materials and Methods

Care and use of animals

Beagles and mixed breed female dogs aged 1–5 years with variable reproductive histories were used for this study. The dogs were cared for in facilities and using procedures that exceed the standards established by the American Association for Accreditation of Laboratory Animal Care. The dogs were purchased from Class A dealers and were kept in quarantine for 2 weeks before integration into the colony. The dogs were fed commercially prepared balanced diets and maintained individually in temperature controlled, environmentally enriched kennels with visual contact with other dogs. The dogs were exercised, mostly in groups, for a minimum of 1 h per day. Individual positive reinforcement training and housebreaking were provided. At the conclusion of their role in the project, the dogs were spayed or neutered before adoption into carefully selected, private, dog-loving homes.

Acquisition of unfertilized oocytes for nuclear transplantation

Ovulation time in female dogs was estimated using a combination of visual observation for proestrus, vaginal cytology to detect cytological oestrus (McDonald, 1980), serum LH assays to detect the LH surge (ICG status-LH; Symbiotics, San Diego, CA) and progesterone assays (Ovucheck, CVS Ltd, Cambs) to confirm the validity of the LH surge.

Oocyte collections were performed 4–7 days after detection of the LH surge to obtain ovulated oocytes for use as recipient ova for nuclear transfer. Bitches were placed under general anaesthesia and the reproductive tract of each bitch was exposed by a mid-ventral laparotomy using aseptic surgical procedures. The fimbriated end of the oviduct was manipulated by digital massage until extruded (everted) through the bursal slit. If necessary, the slit was expanded using fine scissors. The opening in the fimbriated end of the oviduct was located visually and was cannulated using a flanged intramedic catheter (outside diameter 1.27–1.57 mm; Becton Dickinson and Company, Sparks, MD). The catheter was held in place by a surgical ligature, which was tied using a quick-release square knot just below the flange. The catheter was retracted until stopped by the ligature. The base of the oviduct, just above the uterotubal junction, was visualized using digital pressure to blanch the surrounding tissue and the oviductal lumen, and cannulated using a fine (23–27 gauge) hypodermic needle attached to a syringe filled with embryo collection medium (TL Hepes Solution; BioWhittaker, Walkersville, MD). Approximately 4 ml of collection medium was injected through the lumen of the oviduct, through the catheter and directed into a sterile plastic Petri dish. The catheter was removed from the oviduct, the ends were rinsed into the collection dish and the catheter lumen was flushed using the hypodermic needle. After flushing both oviducts, the abdominal incision was closed using a two-layer closure followed by surgical adhesive on the skin incision.

After flushing the oviducts, unfertilized oocytes were recovered from the Petri dish with the aid of a stereomicroscope and used for nuclear transfer as described below. Alternatively, metaphase II bovine oocytes were purchased (Bomed, Madison, WS) and used as recipient ova for interspecies nuclear transplantation.

Acquisition of nucleus donor cells for nuclear transplantation

Cell nuclei were derived from adult skin cells of a spayed mixed breed female bitch aged approximately 11 years. A small piece of skin, approximately 1 cm × 3 cm, was obtained by surgical biopsy using aseptic procedures. Sections of the subcutaneous tissue were removed and standard tissue culture procedures were used to obtain fibroblasts (Hill *et al.*, 2000). Explants were maintained until they approached 90% confluency, and were then trypsinized and reconstituted at a concentration of 1×10^6 cells ml^{-1}.

Production of cloned embryos by nuclear transplantation

Oocytes were denuded (cumulus cells removed) by agitating them up and down in a mouth pipette in 0.5% (w/v) hyaluronidase solution (Sigma, St Louis, MO) for 5 min and washing three times in 20 mmol Hepes buffered TCM199 l^{-1} (GibcoBRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT) (operating medium). The denuded oocytes were pretreated for enucleation by a 15–20 min incubation in operating medium containing 5 μg Hoechst 33342 ml^{-1} (Sigma) and 7.5 μg cytochalasin B ml^{-1} (Sigma). The oocytes were transferred into drops of operating medium contained within a Petri dish and covered with mineral oil (Sigma). Enucleation was performed by removing the first polar body and a small part of the oocyte cytoplasm containing the metaphase II chromosomes using a fine sharpened pipette (17–19 μm in diameter) mounted on Narshige micromanipulators (Narishige, Tokyo). After enucleation, the oocytes were quickly exposed to UV light and observed using fluorescence microscopy to confirm that the Hoechst-stained metaphase chromosomes had been removed.

Dissociated nucleus donor cells, derived as described above, were placed into the microdrop containing the enucleated oocytes. Micromanipulation was used to insert a single donor cell inside the perivitelline space of each enucleated oocyte, such that the plasma membrane was in close contact with the donor cell membrane. The oocyte–fibroblast couplets were washed twice in fusion medium (Liu *et al.*, 1997) and moved into a fusion chamber. The oocyte–fibroblast couplets were aligned manually and fused using a BTX Electrocell Manipulator 200 as described by Liu *et al.* (1997) (BTX Inc, San Diego, CA).

Dog ova were activated after electrofusion by placing the fused couplets into fusion medium in an electrofusion chamber and application of an electrical pulse as described by Kato *et al.* (1998). This procedure was followed by 5 h incubation in 10 μg cycloheximide ml^{-1} (Sigma) and 5 μg cytochalasin B ml^{-1} (Sigma) in operating medium maintained at 39°C. When bovine recipient ova were used, oocyte activation was induced by a 4 min incubation in 5 μmol ionomycin l^{-1} (Sigma) followed by a 5 h incubation in 10 μg cycloheximide ml^{-1} (Sigma) and 5 μg cytochalasin B ml^{-1} (Sigma). After activation, fusion was assessed using a light microscope. The fused couplets were washed three times to remove the cycloheximide completely and placed into a B2-Vero cell monolayer (ATCC, Manassas, VA) coculture system for 2–3 days in 5% CO_2 at 39°C under oil. The resultant cloned embryos were removed periodically from the incubator to monitor development. Embryos developing normally, as judged by cleavage division, were either fixed and stained for cell counts, or were transferred into the reproductive tract of surrogate bitches for *in vivo* development to term (Kraemer *et al.*, 1979, 1982).

Results

The results of experiments involving the transfer of canine cell nuclei into canine oocytes are shown (Table 1). Since October 1999, oocyte collections performed on 17 bitches resulted in 109 ova, of which 63 were judged to be of good enough quality for use as recipients for nuclear transfer. Of these, 61 ova were enucleated successfully and 43 (70%) were fused with canine fibroblasts. Ten of the ova that were fused with canine fibroblasts cleaved to the two- to six-cell stage after 1 or 2 days of culture *in vitro* (Fig. 1). Five of the embryos were transferred into the oviducts of three different recipient females, but no pregnancies resulted. The other five embryos ceased to develop in culture and were fixed and stained to confirm that the blastomeres contained nuclei.

The results of experiments involving the transfer of canine fibroblast nuclei into enucleated bovine oocytes are shown (Table 2). A total of 745 oocytes were enucleated and combined with canine cells. Of these, 291 (39%) were fused successfully. A portion ($n = 200$) was placed into *in vitro* culture, of which 38% ($n = 75$) cleaved to the two- to four-cell stage and 43% ($n = 85$) cleaved to the eight- to 16-cell stage (Fig. 2). Forty-seven embryos at the four- to eight-cell stage were transferred into four different recipient bitches. A single conceptus was detected by ultrasonography at day 19 after embryo transfer; however, the pregnancy subsequently failed (Fig. 3).

Table 1. Nuclear transplantation (cloning) in dogs using canine recipient oocytes and canine nucleus donors.

Number of dogs	Total number of oocytes collected (mean per dog)	Number of good oocytes collected (mean per dog)	Number of enucleated oocytes (%)	Number inserted into cells (%)	Number fused (%)	Number cleaved and fused (%)
17	109 (6.4)	63 (3.7)	61 (97)	61 (100)	43 (70)	10 (23)

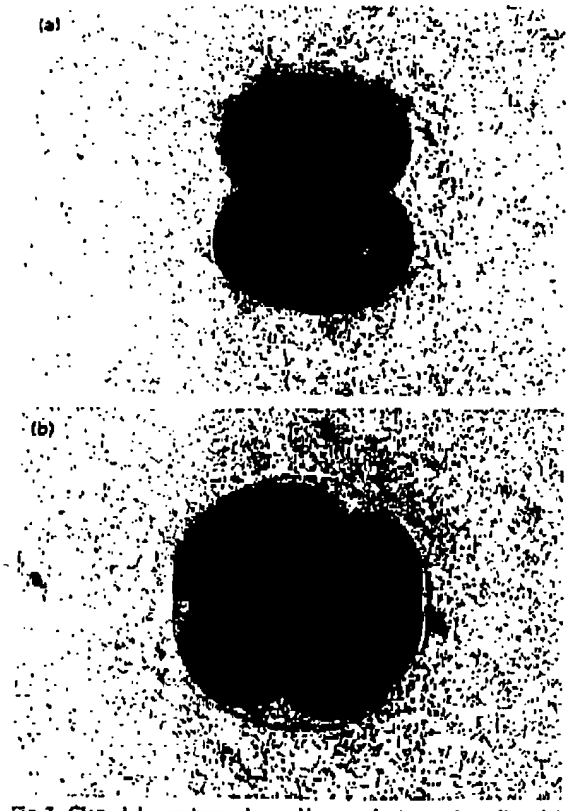

Fig. 1. Cloned dog embryos derived by transferring canine cell nuclei into enucleated canine oocytes. (a) Embryo at the two-cell stage of development. (b) Embryo at the four-cell stage of development.

Discussion

In our laboratory we are exploring the potential for cloning dogs and have made significant progress towards reaching this goal. The basic techniques for nuclear transplantation do not seem to be a limiting factor. Metaphase II oocytes matured *in vivo* can be obtained from dogs using surgical collection techniques. These oocytes can be enucleated and fused with somatic cells obtained from

Table 2. Nuclear transplantation (cloning) in dogs using bovine recipient oocytes and canine nucleus donors

Number of experiments	Number of oocytes enucleated / combined*	Number of oocytes fused with cells	Number of oocytes cultured	Number that cleaved (%)	Number that cleaved to two- to four-cell stage (%)	Number that cleaved to eight- to 16-cell stage (%)
15	745	291 (39)	200	160 (81)	75 (38)	85 (43)

*Number of oocytes enucleated and combined with canine cells.

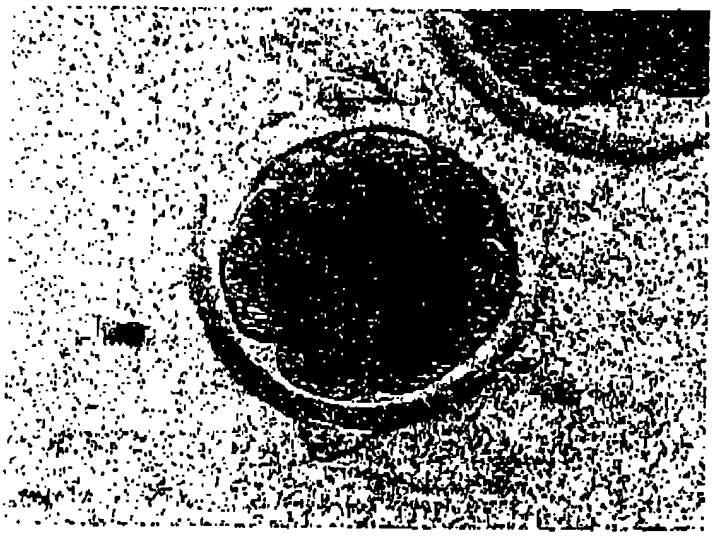

Fig. 2. Cloned dog embryo at the eight- to 16-cell stage of development that was derived by transferring a canine cell nucleus into an enucleated bovine oocyte.

adult animals. The percentage of oocytes that are successfully fused with donor cells is similar to that reported for other species. Of those oocytes that fuse successfully with donor cells, 20–30% undergo at least one cleavage division after artificial oocyte activation and culture *in vitro*. Dog embryos, produced by nuclear transfer, can be transferred surgically into surrogate bitches for development *in vivo*. To date, no pregnancies have resulted from cloned embryos produced by transferring canine nuclei into canine oocytes. However, the number of cloned embryos that have been transferred into surrogate mothers has been extremely limited. Interspecies embryo transfer may be worth pursuing, as indicated by the embryo development obtained using this approach in the present study. However, this tool will probably be more useful in studies to understand basic interactions between oocyte cytoplasm and the transferred nucleus, than for producing offspring.

On the basis of the available information, the most immediate challenge hampering efforts targeted at cloning dogs is obtaining sufficient numbers of high quality metaphase II oocytes to serve as recipients for adult cell nuclei. Oocytes matured *in vivo* can be collected surgically from bitches and used as recipients for nuclear transfer. However, the number of oocytes that can be obtained using this approach is very limited, typically a mean of approximately four good oocytes per collection. Considering the fact that hundreds of nuclear transfer procedures have been required

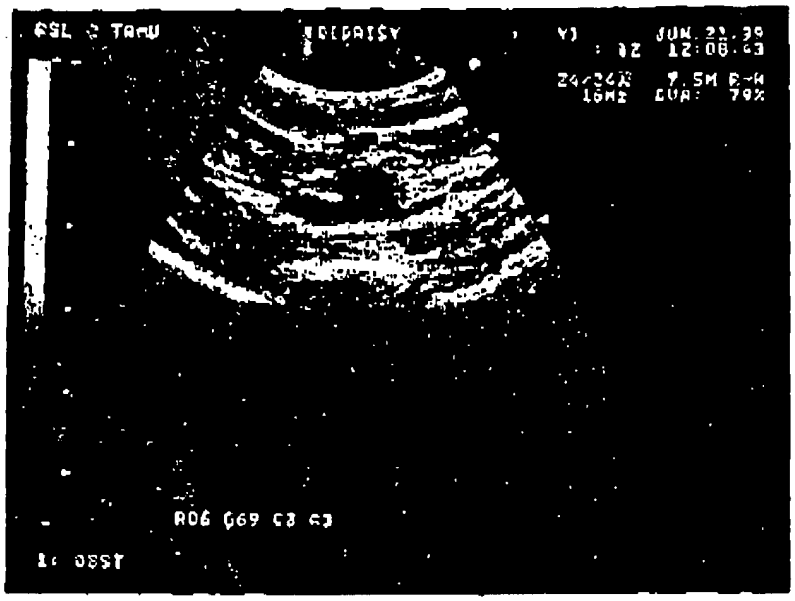

Fig. 3. Ultrasonography of a conceptus obtained after transfer of a cloned dog embryo (derived by transferring a canine cell nucleus into an enucleated bovine oocyte) into a recipient female dog. The hollow appearing structure in the centre of the scan is the conceptus.

in other studies to obtain a single live offspring from other species (Wilmut *et al.*, 1997; Hill *et al.*, 2000), development of methods for obtaining large numbers of unfertilized dog oocytes is of critical importance. Successful techniques for inducing superovulation in bitches by hormonal treatments would be beneficial. However, these techniques have not yet been developed, and even if they were, oocyte numbers would still be limited. Moreover, surgery would still be required to collect the oocytes, and costs for live animals, time and other resources would continue to hamper progress. The most attractive alternative for obtaining large numbers of dog oocytes is *in vitro* maturation. Unfortunately, efforts to develop repeatable methods for obtaining *in vitro* maturation of oocytes collected from anoestrous dogs have so far met with very limited success (Nickson *et al.*, 1993; Yamada *et al.*, 1993; Bolamba *et al.*, 1998; Hewitt *et al.*, 1998; Hewitt and England, 1999).

Whilst the development of methods for obtaining large numbers of mature dog oocytes is currently a major obstacle interfering with efforts to clone dogs, several other factors also represent significant challenges. Synchronization of oestrus, to control the oestrous cycle or cause anoestrous bitches to undergo oestrous cycles, also represents a major challenge. Without this tool, large colonies of dogs are required to assure that females are available to serve as recipients for embryo transfer. Given the long periods of anoestrus in dogs, effective programmes for inducing bitches to ovulate are crucial.

Very few live puppies have been produced after transfer of normal canine embryos into surrogate females (Kraemer *et al.*, 1979, 1982). Therefore, some question remains as to the efficiency of this process. In addition, previous work in this area involved transferring embryos into the uterus of females whose oestrous cycle was naturally synchronous with the embryo donor. No data are available about transfer of dog embryos into the oviducts of synchronized females. If oviductal transfers prove difficult or inefficient, methods for culturing dog embryos from the one-cell stage to a stage at which they can be transferred into the uterus will be required. Although a portion of the

one-cell dog embryos produced by nuclear transfer undergo early cleavage, so far none have developed to the blastocyst stage *in vitro*. To date, there have been no reports of culturing normal dog embryos from the one-cell stage to compact morulae or blastocysts.

Besides the significant technical challenges of developing successful methods for cloning dogs, there are also considerable ethical considerations that must be addressed. Dogs and other companion animals are unique species in terms of their place in society. Many do not place them in the same category as livestock when considering the application of cloning. Therefore, some people who would consider cloning of livestock as completely ethical do not have the same opinion concerning pets. Companion animals, especially dogs and cats, also represent a special situation in terms of animal rights and welfare (Varner, 1999), and their use for scientific research. In this context, considerable effort has been made in this scientific programme to provide a living environment for the dogs which resembles as closely as possible the environment they would have in a private home. In addition, The Missyplicity Project has established strict regulations regarding the treatment of animals. Details can be obtained by reading the 'Code of Bioethics' posted on the Missyplicity website (www.missyplicity.com).

In conclusion, the interest and demand for cloning companion animals will undoubtedly foster the development of successful cloning procedures in dogs. To date, we have received literally hundreds of calls from individuals wanting to clone their pets. Moreover, there are at least four commercial businesses currently offering services that involve cryopreserving cells obtained from pets that might some day be used for cloning. It is difficult to predict when efficient and repeatable methods for cloning dogs and other companion animals will become available; however, it will clearly require a significant commitment to more basic research.

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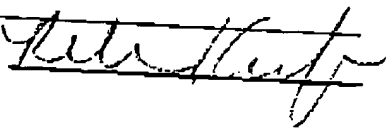
Name: 

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